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Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS): Isolation of Splenic B Lymphocytes

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Overview

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The overall function of the immune system is to defend the body against infectious organisms and other invaders. White blood cells, or leukocytes, are the key players of the immune system. Upon infection, they are activated and initiate an immune response. Leukocytes can be divided into various sub-populations (e.g., myeloid cells, lymphocytes, dendritic cells) based on different parameters that can be biological, physical, and/or functional (e.g., size, granularity, and secretion). One way to characterize leukocytes is through their surface proteins, which are mainly receptors. Each leukocyte population expresses a specific combination of receptors (e.g., cytotoxic, activating, migration receptors) that can define subsets among populations. As the immune system encompasses a wide range of cell populations, it is essential to characterize them to decipher their participation in the immune response.

Flow cytometry (FC or FCM) is a widely used method for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell mixture. Flow cytometers are composed of three main sub-systems: fluidics, optics, and electronics. The fluidics system transports the cells in a stream such that they pass in front of a laser one by one. The optics system consists of light sources (lasers) to illuminate the particles, optical filters to direct the resulting light, and fluorescent signals to appropriate detectors. Finally, the electronics system converts the detected light signals into electronic signals that can be processed by the computer. As an individual cell passes in front of the laser beam, it scatters light. A detector in front of the beam measures forward scatter (FS) and several detectors to the side measure side scatter (SC). The FS correlates with cell size and SC is proportional to the granularity of the cells. In this manner, cell populations can often be distinguished based on differences in their size and granularity alone.

In addition to analyzing a cell's size, shape, and complexity, flow cytometry is widely used for detecting the expression of cell surface receptors (1). This is accomplished by using fluorochrome-labelled monoclonal antibodies which bind to known cell-specific receptors. Upon excitation, these bound fluorochromes emit a light of specific wavelength, called emission wavelength, which can be detected and scored. Fluorescence measurements provide quantitative and qualitative data about fluorochrome-labeled cell surface receptors. Hematologists were first to use FC for the therapeutic follow up of immune cell populations (2). Now, it is used for a wide range of applications such as immunophenotyping, cell viability, gene expression, cell counting, and GFP analysis.

FACS (Fluorescent Activated Cell Sorter) is a specialized type of flow cytometry, which sorts a population of cells into subpopulation using fluorescent labelling. Just like conventional flow cytometry, first FS, SC, and fluorescent data are collected. Then, the machine applies a charge (negative or positive) and an electrostatic deflection system (electromagnets) facilitates the collection of charged droplets containing cells into appropriate tubes.

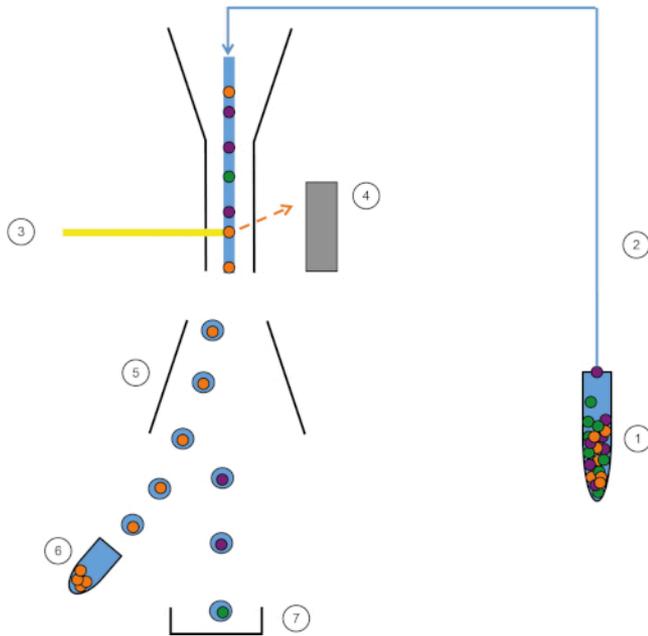


Figure 1: Schematic representation of FACS. Sample (1) is aspirated in the FACS (2) and passed in front of the laser (3). Cell fluorescence is sensed by fluorescence detectors (4). Finally, cells are incorporated in droplets and the cells of interest are deflected by deflection plates (5) and collected in a collection tube (6). The remaining cells go into the trash (7). [Please click here to view a larger version of this figure.](#)

The sorting aspect of the FACS presents many advantages. Many tests can help understand the role of specific cells in immune system, such as analyses of gene expression like RT-qPCR, cell cycle, or cytokine secretion. However, cells should be purified upstream to obtain clear and specific results. Here, FACS comes in useful and the desired cells can be sorted with great purity, yielding highly reliable and reproducible results. FACS can also be used to sort cells based on nuclear or other intracellular staining and according to the presence, absence, and density of surface receptors. FACS is now a standard technique for the purification of subpopulations of cells and has the ability sort up to four populations simultaneously.

This lab exercise demonstrates how to isolate splenic leukocytes and then how to specifically sort B lymphoid cells from the splenic leukocyte cell mixture using FACS.

Procedure

1. Preparation

1. Before beginning, put on laboratory gloves and the appropriate protective clothing.
2. Sterilize all the dissection tools, first with a detergent and then with 70% ethanol and then dry thoroughly.
3. Prepare 50mL of Hank's balanced salt solution (HBSS) containing 2% fetal calf serum (FCS).

2. Dissection

1. Using a carbon dioxide delivery system, euthanize the mouse by hypoxia. Secure the euthanized mouse on a dissection plate in the supine position and perform a longitudinal laparotomy using scissors and forceps.
2. Using forceps, move the intestines and stomach on the right side of the abdomen to expose the stomach and spleen. The spleen is attached to the stomach.
3. Using forceps, carefully detach the spleen from the stomach and place it in the Petri dish containing 5mL of HBSS 2% FCS.

3. Immune Cell Isolation

1. Place the spleen in a 40µm cell strainer over the same Petri dish. Crush the spleen with a plunger to dissociate it in the same dish.
2. Transfer the dissociated spleen and the fluid into a 15mL centrifuge tube.
3. Centrifuge the tube at 370 x g for 7 min at 10°C and discard the supernatant, avoiding the pellet.
4. Resuspend the pellet in 2mL of potassium acetate to lyse the erythrocytes. Wait for 2 min and then make up the volume up to 15mL using HBSS 2% FCS.
5. Centrifuge the tube again at 370 x g for 7 min at 10°C. Discard the supernatant and resuspend the pellet in 5mL of HBSS 2% FCS.
6. Count the cells using a trypan blue staining assay and adjust the final cell concentration to 10^7 cells/mL using appropriate volume of HBSS 2% FCS.

4. Cell Staining

1. Transfer 200µL of the cell suspension into six FACS tubes, labelled 1 to 6.
2. Centrifuge the tubes at 370 x g for 7 min at 10°C and discard the supernatant avoiding the pellet.
3. Next, prepare six novel antibody mixes by adding appropriate amount of antibody to 200µL HBSS 2% FCS according to Table 1.
4. Then, transfer these antibody mixes to the corresponding numbered FACS tubes.
5. Incubate the cell suspensions mixed with the antibodies for 20 minutes on ice in the dark.
6. Add 1mL of HBSS 2% FCS to each tube and then centrifuge again at 370 x g for 3 min at 10°C.
7. Discard the supernatant and resuspend the pellet in 200µL of HBSS 2% FCS.
8. Transfer the resuspended pellets to new FACS tubes.

Mix	Antibodies	Volume	Antibody-dilution
1	None	200µL	
2	Viability-FITC	200µL	1/200
3	CD3-Bv421	200µL	1/100
4	C19-APC	200µL	1/800
5	CD45-APCCy7	200µL	1/1000
6	Viability-FITC, CD3-Bv421, CD19-APC, CD45-APCCy7	200µL	See above

Table 1: Antibodies mix composition. Six mixes of 200µL of HBSS + antibody were prepared for the experiment. Mix 1 is for PMT setting, mixes 2 through 5 are for compensation settings, and mix 6 is for cell sorting.

5. FACS Calibration

1. First, turn on the sorter and perform "**Fluidics Startup**."
2. Turn on the stream and then wait for 15 min for the stream to stabilize.
3. Adjust the amplitude of the stream to obtain detached drop formation. Then click the "**Sweet Spot**" to complete the amplitude adjustment.
4. Put the neutral density (N.D.) filter - 1.0 and open the "**CST**" interface which stands for cytometer setup and tracking.
5. To perform daily quality control, first dilute the CST beads with FACS medium following the manufacturer's instructions and then, perform the CST control.
6. Once CST control is complete, replace the N.D. 1.0 filter with the N.D. 2.0 filter on the cytometer.
7. Next, dilute drop delay beads in FACS medium following the manufacturer's instructions and then load into the FACS.
8. To ensure proper sorting perform **Drop Delay**-
 1. First click "**Voltage**" and then "**Optical Filter**". The right quadrant should be equal to 100%. If necessary, adjust the red laser screw on the cytometer left or right to obtain 100% in the right quadrant.
 2. Next, perform a test sort to ensure that the stream falls into the collection tube. To do so, click on "**Waste Drawer**" and start "**Test Sort**". Check that side streams fall into collection tubes. If they do not, adjust voltage until they do.
 3. Navigate to the experimental template. Then, open the "**Accudrop Drop Delay**" experiment and click the "**Sorting Layout**".
 4. Change the flow rate to obtain 1000 to 3000 events per second.
 5. Click "**Voltage**" and then "**Optical Filter**". The left quadrant should be equal to 0 and right quadrant to 100.
 6. In the "**Sort Layout**" window click on "**Sort**" and then click "**Cancel**". The left quadrant should be equal to 100 and right quadrant to 0. If left quadrant is less than 95 click "**Auto Delay**" to automatically adjust it.

6. Flow Cytometry and Purity Control

1. Begin flow cytometry by starting with the tube 1 (unstained cells) to define the cell morphology and the negative peaks of the fluorochromes. Set up the forward and side scatter and define the voltages of each fluorescent parameter. Place the negative population in the first decade by using the grids on each dot plot.
2. Next, load tube 2 (single color control) in the cytometer. Adjust the spectral overlap until the negative and positive population medians are aligned or use the automatic calculation software. It is important to keep the signal on scale. Compensation controls must match the experimental fluorochromes and detectors settings. Record 10,000 events.
3. Repeat these steps with tube 3, 4, and 5 (other single color controls).
4. Next, load tube 6 (multi-stained cells) and define cell populations of interest by using a specific gating strategy.
5. In Sorting Layout window, select the cell population of interest. Select the cell threshold in the "**Target Events**" and the precision level in the "**Precision**". Here only one population is being sorted, however, four different populations can be sorted at the same time.
6. Once ready click "**Sort**" and "**OK**", then wait for cell sorting.
7. Once cell sorting is complete, perform a purity control by first pipetting 10µL of sorted cells into a new FACS tube with 90 microliters of HBSS 2% FCS.
8. Then, load the tube the cytometer. Record and analyze the phenotypes of the cells to verify that the gating strategy worked as intended.

7. Data Analysis

1. Open the 'FlowJo' software and drag the files for each tube in "**All sample**" window.
2. Double click on a file to open it in a new window.

3. Click on the "Polygon" and recreate gating strategy used previously.
4. Repeat the steps with all the other files.
5. To visualize the dot plots, click on "Layout editor" and drag the populations of interest from tube 6 and purity control in the layout editor tab. Cells should only appear in the population of interest in purity control (see Figure 2).
6. To check the purity of B lymphocytes in the sorted cells, click on "Table editor". Drag B lymphocyte population from tube 6 and purity control in the table.
7. On "Statistic" menu select the frequency of CD45⁺ cells to test the purity of this cell population, then click on "Create table".
8. Parameter values appear in a new table. In purity control window, check the frequency of of B lymphocytes within the CD45⁺ cells, which should be higher than 98% (see figure 2, bottom panel).

Results

In this protocol, we purified splenic B lymphocytes using FACS technology. We first isolated leukocytes from the spleen and stained them. Using a combination of B cell surface markers, we created a gating strategy to sort them (Figure 2, top panel). At the end of the experiment we verified if cells in the collection tube were B cells via a "purity test". We kept the same gating strategy and observed that more than 98% of the cells were indeed B cells (Figure 2, bottom panel). Thus, FACS is an effective protocol to isolate immune cell populations with a high degree of purity. Collected cells can then be used for downstream experiments such as cell culture, RT-qPCR, and cytotoxicity assays.

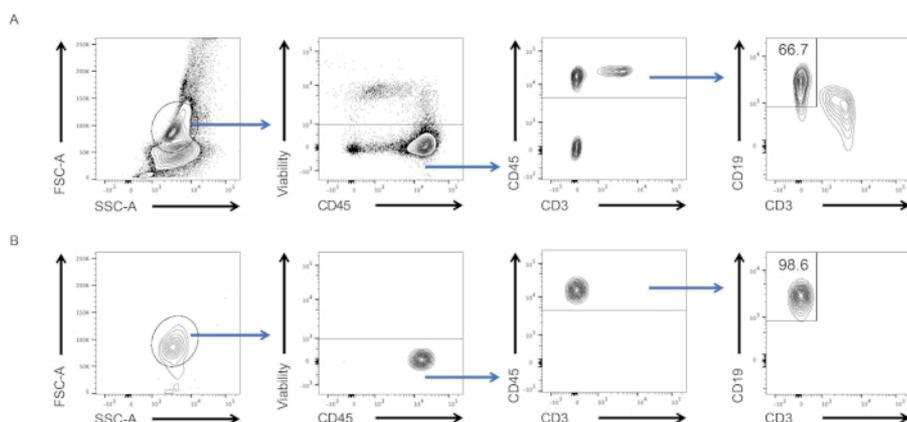


Figure 2: Gating strategy and testing post-sort purity. (A) Cells were first gated based on their morphology (left: FSC-A, SSC-A), then only alive (middle left: viability, CD45), CD45⁺ cells (CD45, CD3) were plotted against CD19 and CD3. Only CD19⁺ cells were sorted. (B) Purity test results of a fraction of cells obtained after cell sorting. [Please click here to view a larger version of this figure.](#)

Applications and Summary

Flow cytometry is a first-hand technique to characterize and sort immune cell populations with a high degree of purity. It is primordial tool in research field as it allows enrichment of specific cell populations and to decipher the immune response to pathogens. With the increase in number of available fluorochromes and cytometers, the number of detectable parameters is highly increased. As a result, bioinformatic analysis of FACS data has begun to emerge and have opened new horizons to flow cytometry (3). Flow cytometry offers other applications in haematology and oncology (4) where it is used for developing diagnostic tools.

References

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